

Involvement of 3-dehydroecdysone in the 3-epimerization of ecdysone

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The epimerization of ecdysone to 3-epiecdysone has been investigated in a dialysed cytosolic enzyme preparation from midgut of sixth instar *Spodoptera littoralis* larvae, with particular emphasis on establishing the intermediacy of 3-dehydroecdysone. Incubation of ecdysone with the dialysed cytosolic preparation furnished 3-dehydroecdysone as the only detectable product, the reaction being oxygen-dependent. The enzyme preparation catalysed reduction of 3-dehydroecdysone to 3-epiecdysone and ecdysone in the presence of NADH or NADPH. Whereas formation of 3-epiecdysone greatly predominated over that of ecdysone in the presence of NADPH, the converse applied when the cofactor was NADH. 3-Epiecdysone incubated with the enzyme preparation in the presence of various cofactors was not metabolized, indicating the irreversibility of the reduction of 3-dehydroecdysone to 3-epiecdysone and, hence, of the 3-epimerization process. The foregoing results, together with comparison of the metabolism of 3-dehydro[³H]ecdysone and [³H]ecdysone by the enzyme preparation in the presence of unlabelled ecdysone and NADPH, support the intermediacy of 3-dehydroecdysone in the 3-epimerization of ecdysone.

INTRODUCTION

During investigation of the transformation, *in vivo* and *in vitro*, of the insect moulting hormones (ecdysteroids), ecdysone and 20-hydroxyecdysone, several metabolic pathways have been detected (for review see Koolman, 1982). Amongst these pathways in some, but by no means all, species investigated is the 3-epimerization process, which converts ecdysteroids (3 β -hydroxy configuration) into their respective 3-epiecdysteroids (3 α -hydroxy configuration). 3-Epi-20-hydroxyecdysone was initially isolated from the meconium of the tobacco hornworm (Thompson *et al.*, 1974), whereas 3-epimerization of ecdysone and 20-hydroxyecdysone was demonstrated *in vitro* in midgut cytosol of that species (Nigg *et al.*, 1974). In addition, 3-epiecdysone (Kaplanis *et al.*, 1979), 3-epi-26-hydroxyecdysone (Kaplanis *et al.*, 1980) and 3-epi-20,26-dihydroxyecdysone (Kaplanis *et al.*, 1979) have been isolated from pupae or developing eggs of *Manduca sexta*, and 3-epi-2-deoxyecdysone has been identified from locust embryos (Isaac *et al.*, 1981a; Tsoupras *et al.*, 1983). Furthermore, several 3-epi[³H]-ecdysteroids have been identified as metabolites in *Pieris brassicae* (Lafont *et al.*, 1980). Since 3-epiecdysteroids are much less active than the corresponding ecdysteroids in the *Musca* bioassay (Nigg *et al.*, 1974; Kaplanis *et al.*, 1979), it is likely that 3-epimerization may be an important process involved in the inactivation of moulting hormones, at least in some species.

Ecdysone oxidase (ecdysone: oxygen 3-oxidoreductase, EC 1.1.3.16), a cytosolic enzyme purified from the blowfly, *Calliphora erythrocephala*, catalyses the transformation of ecdysone and 20-hydroxyecdysone to their corresponding 3-dehydro derivatives (Koolman & Karlson, 1978). It has been suggested that 3-dehydroecdysteroids could be intermediates in the conversion of ecdysteroids into their 3-epimers (Nigg *et al.*, 1974). However, 3-dehydroecdysteroids were not detectable during ecdysteroid 3-epimerization catalysed by an

enzyme preparation from *Manduca* midgut (Mayer *et al.*, 1979). Although 3-dehydroecdysteroids have been reported as metabolites of [³H]ecdysone *in vivo* in a few insect species (for review see Koolman, 1982), a number of these reports merely based on t.l.c. characterization require substantiation, since 3-dehydroecdysteroids co-migrate in such a system with the corresponding 3-acetates (Isaac *et al.*, 1981b). That 3-dehydroecdysone can be reduced in insects is shown by the rapid metabolism of 3-dehydro[³H]ecdysone into ecdysone and/or 3-epiecdysone in *Calliphora vicina* pupae (Karlson & Koolman, 1973; Koolman, 1980) and into both 3-epimers by gut tissues of *Schistocerca gregaria* *in vitro* (Dinan, 1980).

We now report evidence for the intermediacy of 3-dehydroecdysone in the conversion of ecdysone into 3-epiecdysone and establish the cofactor requirements of the enzymes. A cell-free system from midgut of the cotton leafworm, *Spodoptera littoralis*, was used, since 3-epiecdysone and 3-epi-20-hydroxyecdysone have been identified as metabolites of injected [³H]ecdysone and as endogenous compounds in that species (N. P. Milner & H. H. Rees, unpublished work). However, endogenous 3-dehydroecdysteroids were not detected. A part of this work has been presented in preliminary form (Milner & Rees, 1983).

MATERIALS AND METHODS

Insects

Spodoptera littoralis (Boisd.) larvae were reared on an artificial agar-based diet (McKinley, 1970) under licence from the Ministry of Agriculture Fisheries and Food. The insects were maintained at 25 °C and 65–70% relative humidity, and kept under 16 h 'daylight' and 8 h 'dark' periods. Larvae were synchronized (± 2 h) at the fifth-to-sixth larval instar moult, the sixth instar lasting 6 days under the foregoing conditions.

Chemicals

[23,24-³H]Ecdysone (53.6 Ci/mmol) was obtained from New England Nuclear, and ecdysone was from Dr. G. B. Russell, D.S.I.R., Palmerston North, New Zealand. 3-Dehydroecdysone and 3-epiecdysone were prepared as described previously (Dinan & Rees, 1978). H.p.l.c.-grade solvents were purchased from Rathburn Chemicals, Walkerburn, Scotland, U.K.

Enzyme preparation

In a typical preparation of enzyme, the midguts of 12 3-day-old sixth instar larvae were dissected in ice-cold insect Ringer solution (Bodenstein, 1946). The midguts were then thoroughly rinsed in Ringer solution and homogenized in 0.15 M-KCl by 25 passes of a Potter-Elvehjem homogenizer, to give a 20% (w/v) homogenate. The homogenate was centrifuged (10000 *g*) for 5 min in a microcentrifuge, the pellet rehomogenized in 0.15 M-KCl and then centrifuged as before. The supernatants were combined and centrifuged at 100000 *g* for 1 h, to yield the gut cytosol fraction. The cytosol preparation was then dialysed three times for 8 h each against 0.2 M-Tris/HCl buffer, pH 7.5.

Incubation conditions

Incubation mixtures (300 μ l) contained enzyme preparation (150 μ l), ³H-labelled substrate dissolved in 0.2 M-Tris/HCl buffer, pH 7.5 (1 μ g of ecdysteroid per incubation), and appropriate inhibitors or cofactors (in 100 μ l of Tris buffer) to yield a final concentration in the incubation of 2.6 mM for each cofactor. All reactions (in duplicate) were incubated for 1 h at 35 °C in a reciprocating water bath.

Extraction of ecdysteroids

Reactions were terminated by addition of chilled ethanol (750 μ l). The mixture was then centrifuged for 10 min in a bench centrifuge, the supernatant collected, the pellet re-extracted twice with methanol and the combined alcoholic extracts evaporated to dryness under vacuum.

Identification and assay of reaction products

The products of incubation with the midgut enzyme preparation were analysed by h.p.l.c. on a Waters instrument (Waters Associates, Northwich, Cheshire, U.K.) linked to a Waters model 440 u.v.-detector set at 254 nm. Radioactivity was monitored by collecting 1 ml eluent fractions for radioassay. For routine assays, ecdysteroids were chromatographed on an aminopropyl-silica column (APS-Hypersil; 25 cm \times 4.6 mm internal diameter; Shandon Southern Products, Runcorn, Cheshire, U.K.) eluted at a flow rate of 2 ml/min with methanol/dichloroethane [6:96 (v/v); system 1]. This system provides good separation of ecdysone, 3-epiecdysone and 3-dehydroecdysone (Dinan *et al.*, 1981). For additional identification of the reaction products they were chromatographed on a reversed-phase column (Ultrasphere-ODS; 15 cm \times 4.6 mm internal diameter; Beckman-RIIC, Warrington, Cheshire, U.K.) eluted at a flow rate of 1 ml/min with a linear gradient (20 min) of methanol/water (2:3, v/v) changing to methanol/water (4:1, v/v), (system 2; Isaac *et al.*, 1982).

When fractions were collected from the reversed-phase column for radioassay on a liquid-scintillation spectro-

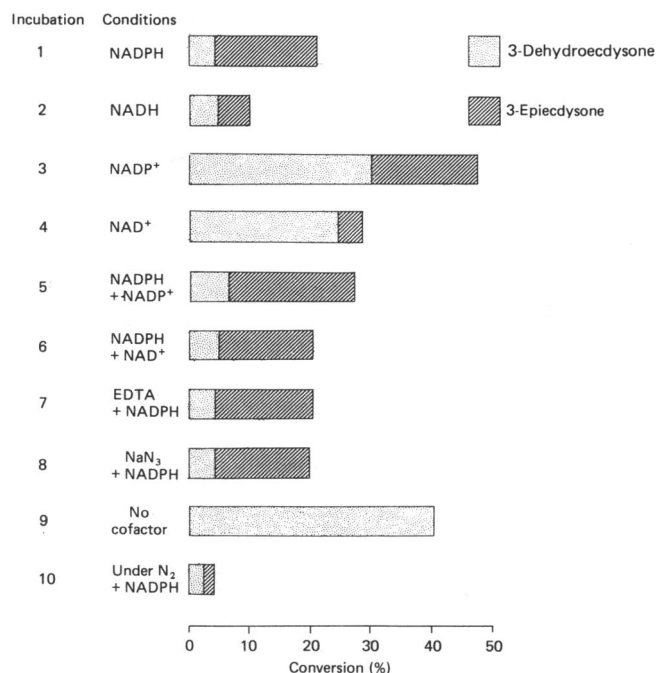


Fig. 1. Conversion of [³H]ecdysone into 3-dehydro[³H]ecdysone and 3-epi[³H]ecdysone in a dialysed midgut cytosol preparation under various conditions

The remaining radioactivity was present in [³H]ecdysone. [³H]Ecdysone (0.25 μ Ci; 0.116 Ci/mol) was incubated in 0.2 M-Tris/HCl buffer, pH 7.5 with dialysed midgut cytosol preparation (150 μ l) in the presence of cofactors or inhibitors as appropriate (NAD⁺, NADP⁺, NADH, and NADPH each at 2.6 mM; EDTA, 20 mM; NaN₃, 1 mM; total incubation volume 300 μ l).

meter (Beckman model LS 9800), scintillation fluid (Scintran Cocktail T, BDH; 4 ml) was added directly without evaporation of solvent. In the case of fractions collected from the APS-Hypersil column, owing to the quenching effect of dichloroethane, the solvent was evaporated to dryness before addition of scintillation fluid.

Preparation of 3-dehydro[³H]ecdysone and 3-epi[³H]ecdysone

3-Dehydro[³H]ecdysone and 3-epi[³H]ecdysone for use as substrates were prepared by incubation of [³H]ecdysone (diluted with unlabelled material to 116 Ci/mol) with *S. littoralis* sixth instar midgut cytosol preparation (dialysed) either in the absence of cofactors or in the presence of NADPH (2.6 mM), respectively. The required products were then purified by successive h.p.l.c. on reversed-phase and APS-Hypersil columns.

Reduction of 3-dehydro[³H]ecdysone with NaBH₄

Putative 3-dehydro[³H]ecdysone dissolved in dry ethanol/tetrahydrofuran [1:1 (v/v), 1 ml] was treated with NaBH₄ (1 mg) and the mixture was left at room temperature for 10 min. The reaction was then stopped by the addition of 1 drop of glacial acetic acid, the mixture was evaporated to dryness under N₂ and redissolved in methanol prior to analysis by h.p.l.c.

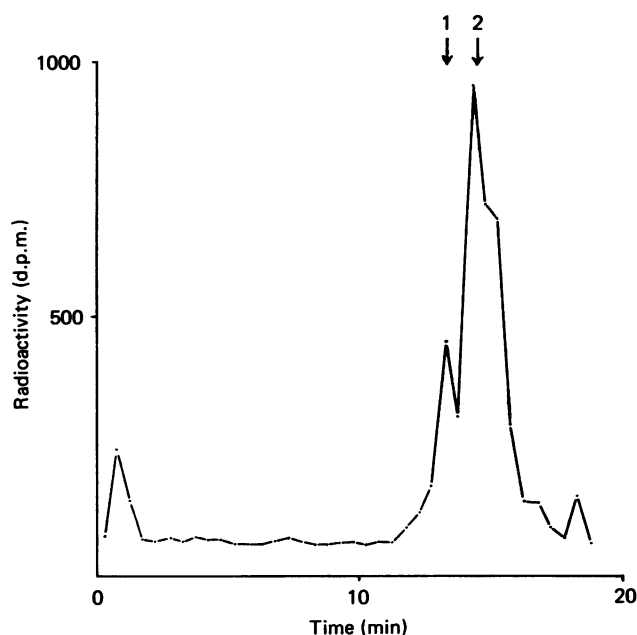


Fig. 2. Reversed-phase h.p.l.c. analysis (system 2) of putative 3-dehydro ^3H ecdysone collected from h.p.l.c. of incubation products on an APS-Hypersil column (system 1)

Arrows indicate the positions of authentic ecdysone (1) and 3-dehydroecdysone (2).

RESULTS

Metabolism of ecdysone to 3-dehydroecdysone and 3-epiecdysone

Preliminary work indicated that no detectable 3-dehydroecdysone accumulated during the transformation of ^3H ecdysone to 3-epiecdysone by a cytosol preparation of *Spodoptera littoralis* sixth instar midgut. Therefore, in an attempt to detect the intermediacy of 3-dehydroecdysone in this transformation, the conversion of ecdysone into 3-epiecdysone was examined in a dialysed cytosol preparation and the effects of cofactors and metalloenzyme inhibitors examined. In addition, the enzyme preparation was also incubated anaerobically following successive evacuation and refilling the incubation tube with N_2 . Details of the incubations and the results are given in Fig. 1. It is apparent that radioactivity cochromatographing with 3-dehydroecdysone on an APS-Hypersil h.p.l.c. column is detectable in several of the incubations. Metabolism of ^3H ecdysone was not detectable in incubations containing boiled enzyme.

Characterization of 3-dehydro ^3H ecdysone and 3-epi ^3H ecdysone

To confirm the identity of the 3-dehydro ^3H ecdysone produced by incubation of ^3H ecdysone with the dialysed midgut cytosol enzyme preparation, further characterization was necessary. The radioactivity corresponding to 3-dehydroecdysone on the APS-Hypersil h.p.l.c. column (system 1) was collected and rechromatographed on a reversed-phase column (system 2) together with authentic 3-dehydroecdysone. The results shown in Fig. 2 indicate the presence of a major peak of radioactivity cochromatographing with 3-dehydroecdysone.

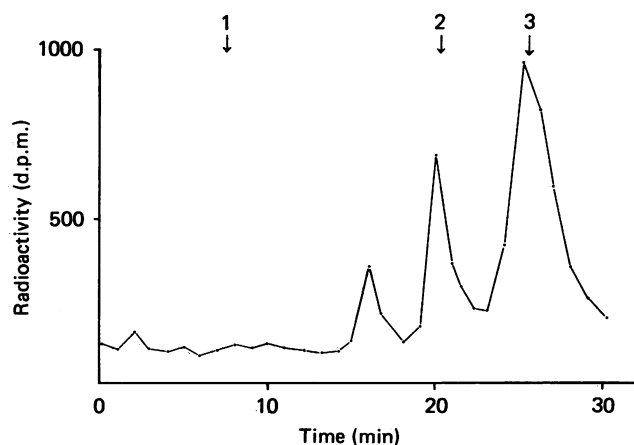


Fig. 3. H.p.l.c. analysis (APS-Hypersil; system 1) of the products of reduction of putative 3-dehydro ^3H ecdysone with NaBH_4

Arrows indicate the positions of authentic 3-dehydroecdysone (1), ecdysone (2) and 3-epiecdysone (3).

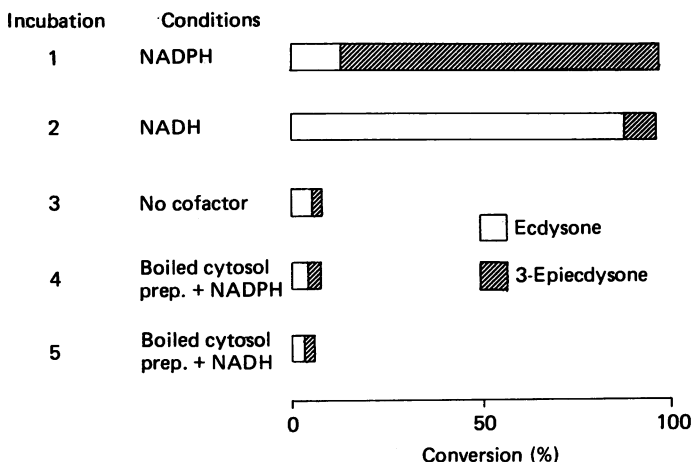


Fig. 4. Conversion of 3-dehydro ^3H ecdysone into ^3H ecdysone and 3-epi ^3H ecdysone in a dialysed midgut cytosol preparation under various conditions

The remaining radioactivity was present in 3-dehydro ^3H ecdysone. When present, NADPH and NADH were each added at 2.6 mM concentration.

The putative 3-dehydro ^3H ecdysone was also subjected to reduction with NaBH_4 and the products of the reaction were analysed by h.p.l.c. on APS-Hypersil (system 1; Fig. 3). The chromatogram reveals the presence of two major peaks of radioactivity corresponding to ecdysone and 3-epiecdysone as expected, together with a smaller unidentified peak.

3-Epi ^3H ecdysone was characterized by cochromatography with authentic material on both reversed-phase (system 2) and APS-Hypersil (system 1) h.p.l.c. columns. In addition, as expected, the 3-epi ^3H ecdysone would not form an acetonide derivative by the method of Galbraith & Horn (1969).

For additional characterization of 3-dehydroecdysone and 3-epiecdysone by mass spectrometry, large scale

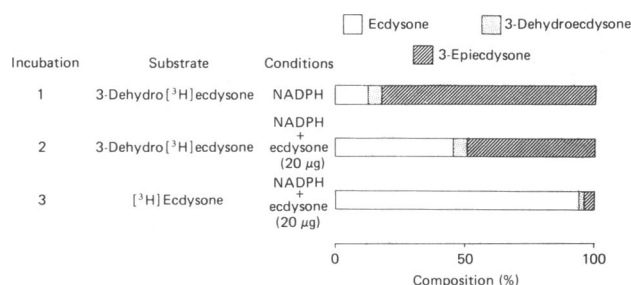


Fig. 5. Composition of the reaction products from incubations of 3-dehydro[^3H]ecdysone and [^3H]ecdysone with dialysed midgut cytosol preparation

NADPH was added at 2.6 mM concentration.

incubations of ecdysone were performed. In this case, ecdysone ($30\text{ }\mu\text{g}$) was incubated with 5 ml of dialysed cytosol preparation in the presence or absence of NADPH (2.6 mM), to produce 3-epiecdysone and 3-dehydroecdysone, respectively, which were purified by successive h.p.l.c. on APS-Hypersil and reversed-phase columns. The negative ion fast atom bombardment (FAB) mass spectra, obtained by using a VG 7070 spectrometer employing glycerol as the probe solvent, showed prominent ions corresponding to $(M-H)^-$ at m/z 461 and 463 for 3-dehydroecdysone and 3-epiecdysone, respectively.

Incubation of 3-epi[^3H]ecdysone with midgut cytosol preparation

To establish the reversibility of the ecdysone 3-epimerization reaction *in vitro*, 3-epi[^3H]ecdysone was incubated with (i) undialysed midgut cytosol preparation, (ii) dialysed midgut cytosol preparation both without cofactor and with (iii) NADPH, (iv) NADH, (v) NADP^+ and (vi) NAD^+ . The reaction products were analysed on an APS-Hypersil h.p.l.c. column (system 1). In all cases, only unmetabolized 3-epi[^3H]ecdysone was detected, indicating that the ecdysone 3-epimerization reaction is irreversible.

Incubation of 3-dehydro[^3H]ecdysone with midgut cytosol preparation

Having established that 3-dehydroecdysone is formed from ecdysone, the metabolism of [^3H]3-dehydroecdysone was investigated in dialysed midgut cytosol preparation, with and without cofactor. The results of these incubations (Fig. 4) demonstrate that in the presence of NADPH the reduction of 3-dehydroecdysone to 3-

epiecdysone is favoured, whereas NADH promotes considerably more reduction to ecdysone.

Intermediacy of 3-dehydroecdysone in the conversion of ecdysone into 3-epiecdysone

Although the foregoing experiments have implied the intermediacy of 3-dehydroecdysone in the 3-epimerization of ecdysone, they do not preclude the possibility that 3-dehydroecdysone and 3-epiecdysone are formed from ecdysone via separate pathways:



To investigate this possibility, 3-dehydro[^3H]ecdysone was incubated with dialysed midgut cytosol preparation in the presence of NADPH, with and without unlabelled ecdysone ($20\text{ }\mu\text{g}$). In addition, [^3H]ecdysone was also incubated with the same cytosol preparation in the presence of unlabelled ecdysone ($20\text{ }\mu\text{g}$) and NADPH.

The proportion of reaction products (analysed by h.p.l.c. system 1; Fig. 5) provide evidence that the above reaction sequence does not occur. In the presence of unlabelled ecdysone, conversion to 3-epiecdysone from [^3H]ecdysone (incubation 3) is much less than that observed from 3-dehydro[^3H]ecdysone of the same specific radioactivity (116 Ci/mol) as the initial [^3H]ecdysone (incubation 2).

DISCUSSION

The cytosolic enzyme preparation from *S. littoralis* midgut, in the presence of NAD(P)H , converts 3-dehydroecdysone into both 3-epiecdysone and ecdysone (Fig. 4). Similarly, [^3H]ecdysone is converted into 3-dehydroecdysone and 3-epiecdysone in the presence of NADPH, whilst without cofactor ecdysone is only transformed to 3-dehydroecdysone (Fig. 1). These results provide experimental evidence for the intermediacy of 3-dehydroecdysone in the isomerization of ecdysone to 3-epiecdysone, as originally proposed for 3-epiecdysone formation in *Manduca sexta* midgut (Nigg *et al.*, 1974). In addition, the isomerization reaction has been shown to be irreversible, although 3-dehydroecdysone can be converted into ecdysone as well as 3-epiecdysone. Indeed, a similar metabolic relationship between 20-hydroxyecdysone, 3-dehydro-20-hydroxyecdysone and 3-epi-20-hydroxyecdysone has recently been established in midgut preparations from *Pieris brassicae* larvae (Blais & Lafont, 1984).

The conversion of ecdysone into 3-dehydroecdysone does not require the presence of dialysable cofactor (Fig. 1, incubation 9). This is in contrast to 3 β -hydroxysteroid

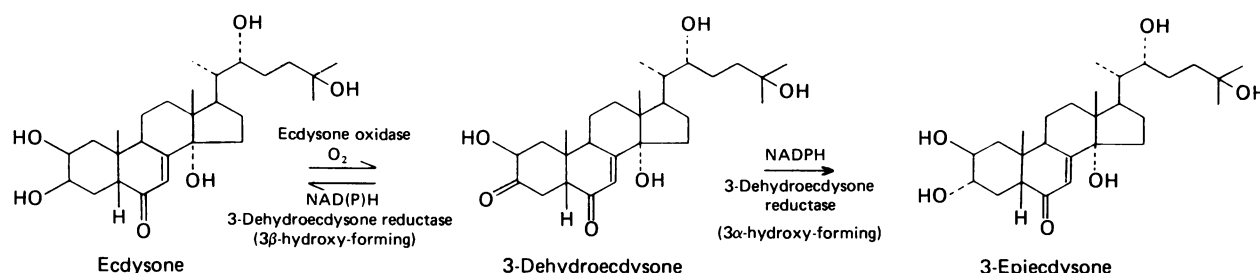
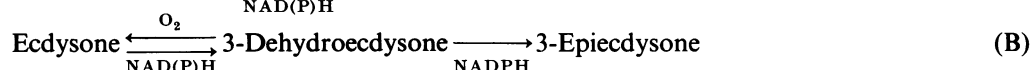
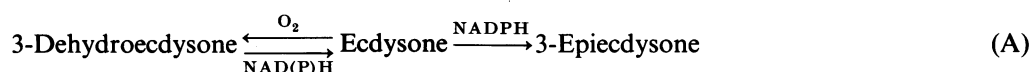


Fig. 6. Proposed metabolic scheme for the production of 3-epiecdysone in *Spodoptera littoralis* midgut

dehydrogenases (EC 1.1.1.51) from vertebrates and micro-organisms, which require NAD(P)⁺ as cofactor. The enzyme involved in the conversion of ecdysone into 3-dehydroecdysone in *Spodoptera* midgut has more in common with the ecdysone oxidase from *Calliphora erythrocephala* (Koolman & Karlson, 1978). Indeed, under N₂ (i.e. in the virtual absence of oxygen) very little ecdysone is converted into 3-dehydroecdysone (Fig. 1, incubation 10), confirming that the enzyme responsible for the formation of 3-dehydroecdysone is indeed an oxidase.

When [³H]ecdysone is incubated in the presence of NADPH or NADH very little 3-dehydroecdysone is isolated (Fig. 1, incubations 1 and 2), most of the intermediate being reduced to 3-epiecdysone and ecdysone. Indeed, in the presence of reduced cofactors, the overall conversion of ecdysone into reaction products is apparently lowered. This is undoubtedly due to the reversible nature of the ecdysone to 3-dehydroecdysone transformation in the presence of reduced cofactors.



NADPH appears to be the most effective cofactor for the reduction of 3-dehydroecdysone to 3-epiecdysone (Fig. 1, compare incubations 1 and 2). In the presence of NADPH, 3-dehydroecdysone is reduced in what appears almost equal proportions to ecdysone and 3-epiecdysone (Fig. 1, compare incubations 1 and 9), whereas in the presence of NADH, 3-dehydroecdysone is preferentially converted into ecdysone rather than 3-epiecdysone (Fig. 1, compare incubations 2 and 9). These findings differ from the ecdysteroid 3-epimerization system from *Manduca* midgut for which NADH and NADPH are, apparently, virtually equally effective (Mayer *et al.*, 1979).

Although 3-dehydroecdysone was not detected during ecdysone 3-epimerization by a *Manduca* midgut enzyme preparation, the transformation was oxygen-dependent (Mayer *et al.*, 1979), suggesting the involvement of an ecdysone oxidase analogous to that found in *Calliphora* (Koolman & Karlson, 1978). In *Manduca* it was proposed that 3-dehydroecdysone may be a very short-lived intermediate (Mayer *et al.*, 1979).

Since the addition of EDTA and NaN₃ has no apparent effect on the overall conversion of ecdysone into 3-dehydroecdysone and 3-epiecdysone (Fig. 1, compare incubations, 1, 7 and 8), the involvement of a metalloenzyme in this transformation is very unlikely. This is in agreement with previous findings for ecdysone oxidase from *Calliphora* (Koolman & Karlson, 1978). The addition of the oxidized cofactors, NAD⁺ and NADP⁺, to the enzyme incubations produced somewhat surprising results, 3-dehydroecdysone being reduced to 3-epiecdysone (Fig. 1, incubations 3 and 4). This phenomenon, also encountered with *Manduca* midgut preparations (Mayer *et al.*, 1979) is most probably due to the reduction of some NAD(P)⁺ to NAD(P)H in the relatively crude enzyme preparation employed in this study. Presumably, only small amounts of NADH or NADPH are produced in this way, but sufficient quantities are present to convert 3-dehydroecdysone into 3-epiecdysone in similar amounts to those observed in

incubations 1 and 2. However, it is clear that reduced cofactors produced in incubations 3 and 4 are not in sufficient concentration to reduce maximally 3-dehydroecdysone back to ecdysone, as witnessed by the increased levels of 3-dehydroecdysone in these incubations compared with those occurring in the presence of reduced NAD(P)H (incubations 1 and 2). This interpretation of the effect of NAD⁺ and NADP⁺ is supported by the reaction products obtained in incubations conducted in the presence of NAD⁺ or NADP⁺ in addition to NADPH (Fig. 1, incubations 5 and 6). In these cases, the presence of NAD⁺ or NADP⁺ does not significantly modify the reaction products (compare with incubation 1).

Owing to the reversible nature of the transformation of ecdysone to 3-dehydroecdysone, mere incubations of either [³H]ecdysone or 3-dehydro[³H]ecdysone cannot distinguish between two possible metabolic relationships shared by ecdysone, 3-dehydroecdysone and 3-epiecdysone:

Incubation of [³H]ecdysone in the absence of oxygen (Fig. 1, incubation 10) virtually inhibits formation of 3-dehydroecdysone and 3-epiecdysone, strongly suggesting that reaction sequence B is operating. Confirmation of this supposition was obtained by the incubation of radioactive substrates ([³H]ecdysone or 3-dehydro[³H]ecdysone of equal specific radioactivities) in the presence of unlabelled ecdysone 'trap' (Fig. 5). If sequence A was operating, formation of 3-epi[³H]ecdysone would be expected to be less in incubation 2 than in 3; this was not the case. Indeed, as incubation 2 (Fig. 5) yields more 3-epi[³H]ecdysone than incubation 3, it is virtually certain that reaction pathway B is correct. Although it is possible that direct 3-epimerization of ecdysone may occur simultaneously with the reaction via 3-dehydroecdysone, no evidence to support this has transpired in the foregoing experiments.

Less 3-epi[³H]ecdysone was formed in incubation 2 than in incubation 1 (Fig. 5) presumably because any 3-dehydro[³H]ecdysone converted into ecdysone in the former was diluted with unlabelled ecdysone and, therefore, tended to be 'trapped' as ecdysone rather than be reconverted into 3-dehydroecdysone and 3-epiecdysone. In incubation 1, 3-dehydro[³H]ecdysone reduced to ecdysone was presumably actively reconverted into 3-dehydroecdysone and, therefore, on to 3-epiecdysone.

The incubation of 3-dehydro[³H]ecdysone with and without ecdysone 'trap' (compare incubations 1 and 2 in Fig. 5) allows reduction to ecdysone and 3-epiecdysone in the presence of NADPH to be estimated. In incubation 2 any [³H]ecdysone formed is diluted by unlabelled ecdysone, so that the extent of the reduction of 3-dehydroecdysone to ecdysone can be estimated. Indeed, in the presence of NADPH almost equal reduction to ecdysone and 3-epiecdysone apparently occurs (Fig. 5, incubation 2), confirming the earlier conclusion (Fig. 1, incubation 1).

The combined results support the overall metabolic scheme shown in Fig. 6 for the 3-epimerization of

ecdysone, implicating the likely involvement of ecdysone oxidase and a 3-dehydroecdysone reductase (3 α -hydroxy-forming) in this transformation.

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